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Volume-dependent spatial distribution of microinjected thyrotropin-releasing hormone (TRH) into the medial preoptic nucleus of the rat: an autoradiographic study

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The present study was performed to quantify the distribution of a peptide neurotransmitter after microinjection into the medial preoptic area (POM), using a technique suitable for conscious animal preparations. The results indicate that only 50-nl volumes of injected tracer were sufficiently localized with $77 \pm 9\%$ recovery in the POM. Injections of higher volumes resulted in an increasing spread of tracer into distant anatomical regions and structures, including the needle tract and cerebral ventricles. The amount of tracer localized in the POM decreased to $38 \pm 4\%$ (200 nl) ($P < 0.05$) and $41 \pm 8\%$ (500 nl) ($P < 0.05$), respectively. The data suggest that the volume of injection is critical for intraparenchymal injections into structures of a diameter of 1 mm or less, such as the POM and should not exceed 50 nl in conscious animal preparations.

Microinjections of neurotransmitters and drugs into discrete areas of the brain have been a valuable tool in the neurosciences to study physiological functions of neuronal clusters [11]. In order to deliver small doses and/or volumes in nanoliter quantities, investigators usually turn to the use of glass micropipettes and techniques such as pressure injection or iontophoresis (for review see [1] and [11]). These arrangements, however, require that the animal be anesthetized. Hence, in order to omit the effects of anesthetics, especially in studies of central autonomic and behavioral functions, the necessity of conscious, unrestrained animal preparations is evident, which disallows the use of fragile glass micropipettes. Due to their larger size, however, metallic cannulas generally require higher injection volumes, which may spread

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14. ABSTRACT

The present study was performed to qua ntify the distribution of a peptide neurotransmitter after microinjection into the medial preoptic area (POM), using a technique suitable for conscious animal preparations. The results indicate that only 50-ni volumes of injected tracer were sufficiently localized with 77 ? 9% recovery in the POM. Injections of higher volumes resulted in an increasing spread of tracer into distant anatomical regions and structures, including the needle tract and cerebral ventricles. The amount of tracer localized in the POM decreased to 38?4% (200 nl) (P < 0.05) and 41 ?8% (500 nl) (P <0.05), respectively. The data suggest that the volume of injection is critical for intraparenchymal injections into structures of a diameter of I mm or less, such as the POM and should not exceed 50 nl in conscious animal preparations.

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more diffusely in the target area, thus potentially leading to a widespread rather than a localized neuronal response [6].

Particular interest in our laboratory has been drawn to the involvement of thyrotropin-releasing hormone (TRH) in the hypothalamic mediation of cardiovascular responses to environmental stress. We have shown that microinjections of TRH into the medial preoptic area (POM) of conscious, unrestrained rats elicit cardiovascular changes in a pattern similar to the classical defense reaction involving vasodilatation in the hindquarters and constriction in renal and splanchnic vasculature [8, 9].

Although radioactive tracers have been used to determine the localization of a compound microinjected into discrete brain regions [2, 3, 10], no attempt was made so far to quantify the spread of different volumes of drug solutions after local injections in conscious animal models. We therefore undertook the present study using an autoradiographic technique to determine the spread of [^{3}H][3Me-His^2]-TRH, a TRH analog [12], upon microinjection of different volumes into the POM.

Male Sprague–Dawley rats were obtained from Taconic Farms (Germantown, NY) and housed at controlled temperature (22°C) and light cycle (12 h/12 h). Seventeen rats were used for the study. The animals were anesthetized with an intramuscular injection of ketamine (130 mg/kg) and acepromazine (1.2 mg/kg) and stainless steel guide cannulas were implanted over the right parietal skull (−0.3 mm ap, −0.8 mm lat from bregma) prior to the experiment. Radioactive tracer ([^{3}H][3Me-His^2]TRH in ethanol, specific activity 55 Ci/mmol, New England Nuclear) was injected into the right POM with a 500-nl Hamilton microsyringe connected to a 30-gauge cannula by PE50 tubing. The length of the cannula was 9.5 mm. Volumes of 50, 200 or 500 nl of a constant tracer concentration (15 μM) were administered manually over 30 sec. Two minutes after the injection the animals were sacrificed, the forebrains immediately removed, and rapidly frozen on powdered dry ice; this procedure did not exceed 1 min. This time period was chosen since maximal changes of cardiovascular parameters and sympathetic nerve activity occurred within 2 min after injection of TRH into the POM. 10-μM sections were cut in a cryostat at −10°C, thaw mounted onto microscope slides, and rapidly dried on a hot plate. The slides were placed together with tritiated plastic standards (^{3}H-microscales, Amersham), in X-ray cassettes and exposed to LKB Ultrofilm (LKB, Gaithersburg) for 4 weeks at 6–8°C. The films were then developed in D-19 Developer (Kodak, 15–18°C) for 5 min and fixed with GBX Fixer and Replenisher (Kodak) for 10 min. Quantitative analysis of injection area/section and amount of tracer/section was done by means of computer-assisted densitometry (RAS-3000 system, Amersham). Two different magnifications (1:25 and 1:5.5) were used to measure the tracer distribution in the histologically verified center of the injection at the tip of the injection cannula and the overall spread of radioactive material, respectively. In order to obtain percentage values of tracer volume localized in the POM vs. overall spread, the distribution of [^{3}H][3Me-His^2]TRH within the POM was also determined at the lower magnification. Volumes were calculated as curve areas according to a trapezoidal method. Curve areas were thereby approximated by summation of various trapezoids fitted to different curve segments. A nonparametric statistical test (Kruskal–Wallis) [5] was

used to compare the resulting volumes. Data are expressed as means $\pm$ SE.

Representative examples of tracer injections and histologic location are shown in Fig. 1 for the groups with 50 nl ($n=8$), 200 nl ($n=3$) and 500 nl ($n=6$) injections. The volume of tracer found in the target area ('injection tip'), as verified by the histological location of the needle tip, increased from 1.1 ± 0.2 mm^3 in the 50-nl group to 4.3 ± 1.5 mm^3 (200 nl, $P<0.05$) and 4.0 ± 1.2 mm^3 (500 nl, $P<0.05$) (magnification 1:25, Fig. 2A). Correspondingly, the overall spread of tracer as defined as tracer localized in the injection tip, needle tract, and ventricles was 5.0 ± 1.1 mm^3, 20.2 ± 2.0 mm^3 ($P<0.05$ vs. 50 nl) and 20.1 ± 6.3 mm^3 ($P<0.05$ vs. 50 nl), respectively (magnification 1:5.5, Fig. 2A). Fig. 2B demonstrates the sagittal distribution vs. the area/sec-

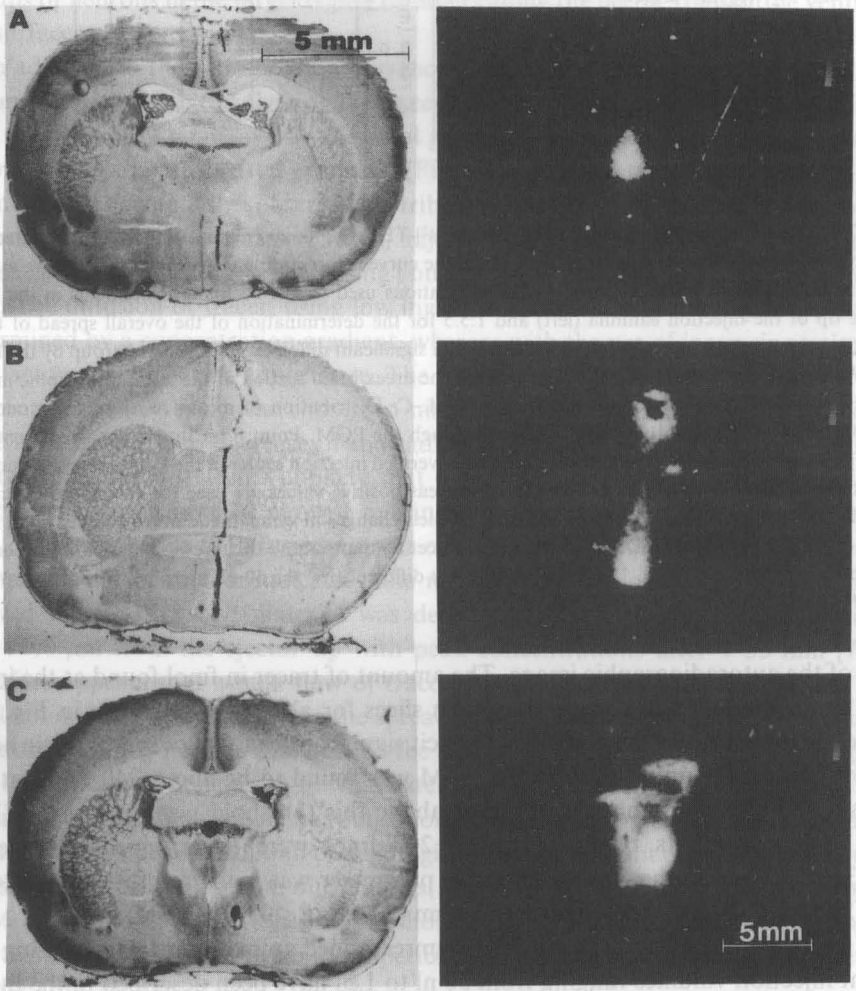

Fig. 1. Localization of microinjections of [^{3}H][3Me-His^2]-TRH into the POM. The histological verification of the locus of the injection in thionine-stained brain sections (left, scale 1:8.8) is compared to the autoradiographic distribution of tracer (right, scale 1:5.2) in an adjacent slice. The upper row (A) represents injections of 50 nl of [^{3}H][3Me-His^2]-TRH, (B) 200 nl, and (C) 500 nl, respectively.

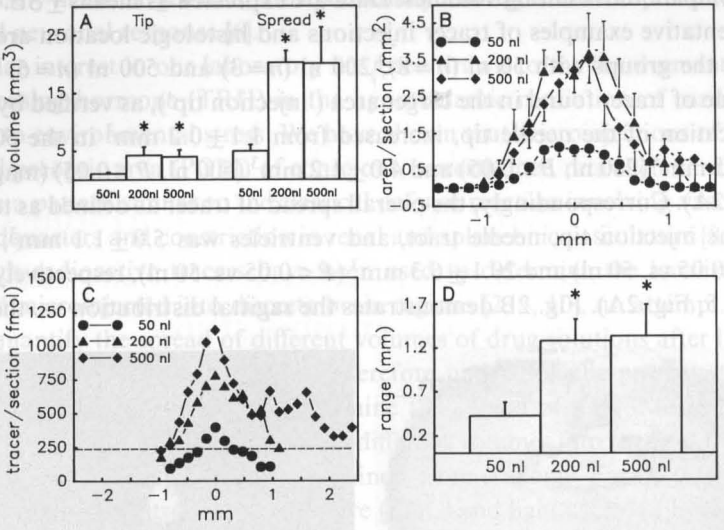

Fig. 2. A: Tissue volumes occupied by [^{3}H][3Me-His^2]-THR (50 nl, 200 nl, and 500 nl) microinjected into the POM. The volumes are calculated as areas of the curves representing the areas of consecutive autoradiographic images of serial sections. The magnifications used were 1:25 for measurements in the POM at the tip of the injection cannula (left) and 1:5.5 for the determination of the overall spread of tracer including tip, needle tract and ventricles. *Denotes a significant difference vs. the 50-nl group by the Kruskal–Wallis test, $P<0.05$. B: Serial distribution of the areas/brain section of the autoradiographic images of [^{3}H][3Me-His^2]-TRH contained within the POM. C: Distribution of means of absolute amounts of [^{3}H][3Me-His^2]-TRH (fmol) in serial sections through the POM. Point 0 on the abscissa represents the peak amount of tracer detected in the histologically verified injection center in the POM, negative numbers denote serial sections in the caudal direction, whereas positive values are used for the rostral extension. The dashed line symbolizes the threshold dose to elicit changes in sympathetic nerve activity upon injections of TRH into the POM. D: Sagittal range of tracer amounts above the threshold dose to elicit changes of sympathetic nerve activity. *Denotes a significant difference vs. the 50-nl group by the Kruskal–Wallis test, $P<0.05$.

tion of the autoradiographic image. The amount of tracer in fmol found at the injection tip in the POM in consecutive brain slices for all groups is shown in Fig. 2C. Since the lowest active dose of TRH to elicit significant changes of sympathetic nerve activity after microinjection into the POM was found to be about 240 fmol/rat [8], the sagittal range of amounts of tracer above this threshold was determined (Fig. 2D). While amounts of tracer above 240 fmol were found over a range of 428.3 ± 151.8 μm in the 50-nl group, this parameter was increased to 1280.0 ± 244.4 μm (200 nl, $P<0.05$) and 1333.3 ± 359.4 μm (500 nl, $P<0.05$ vs 50 nl).

Microinjections of drugs in conscious, unrestrained animal preparations using different injection volumes ranging from 50 nl to 1 μl have been described in the literature [2, 9]. The results of the present study indicate that injections of increasing volumes of a radioactive tracer, [^{3}H][3Me-His^2]-TRH, into the POM, an area, which extends over 1–1.5 mm rostro-caudally and 1 mm laterally on each side of the third ventricle [4], lead to an increased deposition of tracer outside the target area. While

injections of 50 nl appear to be distinctly localized with about $77 \pm 9\%$ of the overall detected radioactivity contained in the injection tip within the POM, higher volumes, largely spread through the needle tract and the ventricles with only $38 \pm 4\%$ (200 nl) ($P < 0.05$) and $41 \pm 8\%$ (500 nl) ($P < 0.05$) of the tracer volume detected in the POM. Although thaw mounting and consecutive drying of the frozen sections may result in a slight overestimation of the absolute volumes in general, this is unlikely to account for the striking differences in the spread of tracer between the 50 nl and 200 nl and 500 nl group, respectively (Fig. 1). Thus, dependent on the dose and volume of an administered drug careful considerations have to be drawn, whether the biological effects observed after local injections are indeed attributable to excitation of groups of neurons in the target region or others along the needle tract or the ventricular surfaces.

In addition to the widespread occurrence of tracer material outside the POM, the volume of the injectate at the tip of the needle increased by about 4-fold in the 200-nl and 500-nl groups compared to the 50-nl group. This appeared to be mainly due to lateral, rather than to sagittal extension (Fig. 2B). Since the tracer concentration was maintained constant, the observed distribution may be solely attributable to the increased volume factor. In contrast, injection of a constant dose in increasing volumes, would introduce a decreasing concentration gradient as an additional variable. The diffusion of tracer, using low injection volumes, may thereby be primarily determined by a concentration gradient, whereas with the use of increasing volumes, this factor may be of less importance. The regimen of injections of a constant tracer concentration, however, was chosen to maintain the signal intensity, which would have been expected to decrease, if the radioactive source would have been diluted. On the other hand it seems unlikely that especially the sagittal distribution of the tracer, which appears to be above 1 mm in both directions from the injection center (Fig. 2B) is solely due to diffusion along a concentration gradient. Studies on the diffusion capacity of smaller molecules like normetanephrine and 3-methoxytyramine showed that the injected material was detectable after 3–6 min in a distance of 650–1000 μm from the ejector tip with peak concentrations after 4–35 min [7]. It therefore appears that a bulk flow of tracer driven by a pressure gradient due to a local volume increase may add to the spread of the injected material. However, since there are currently no data available on the diffusion coefficient of TRH, it is yet difficult to draw a final conclusion to what extent these mechanisms contribute to its distribution after parenchymal microinjection. Even more important than the mere spread is the spatial distribution of a biologically active amount of the injected neurotransmitter or drug. Based on our findings, 240 fmol of TRH, microinjected into the POM of the rat, appears to be the threshold dose to elicit autonomic responses [8]. Our results clearly demonstrate the superior local confinement of the 50-nl injection volume (Fig. 2C,D).

In summary, the present study emphasizes the use of low nl volumes in studies utilizing the technique of microinjections in conscious animal preparations in order to provide an adequate localization of the central nervous origin of biological responses to neurotransmitters or drugs. Caution has to be applied, however, to extrapolate

the presented results to other peptides with different diffusion coefficients or techniques, including different injection rates and concentrations, which may crucially influence the tissue distribution of the injected material. It may therefore be necessary in studies using the microinjection technique, to carefully analyze the spread of an injected compound under the particular experimental conditions.

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or as necessarily reflecting the views of the Department of Defense or the USUHS. The experiments reported herein were conducted according to the principles set forth in the 'Guide for Care and Use of Laboratory Animals', Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. NIH 85-23, 1985) and were supported by USUHS Grant R09232 to Dr. Sirén.

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